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In-Vivo Studies on Anti-Diabetic Potential of *Leucas Aspera* in Streptozotocin Induced Diabetic Wistar Albino Rats

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ABSTRACT

Nanotechnology is being a utilized in medicine for diagnosis, therapeutic drug delivery and for the development of treatment for many ailments and disorders specifically in the areas of drug delivery, as medical diagnostic tools, and as diseases cure agents. During the past decades, the biosynthesis of metal nanoparticles has received considerable attention due to the growing need to develop environmentally sociable technologies in material synthesis. In this study, we investigated the anti-diabetic potential of *Leucas aspera* leaf extract in streptozotocin-induced diabetic Wistar albino rats and, serum creatinine, blood urea, protein content, enzymatic antioxidant, and non-enzymatic antioxidant was estimated. This study evidenced the efficacy of the anti-diabetic potential of *Leucas aspera* leaf extract in the *in-vivo* model.

Keywords: *Diabetes mellitus, Iron oxide nanoparticles, Leucas aspera, Streptozotocin (STZ).*

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INTRODUCTION

Diabetes mellitus is an endocrinal disorder related to depleted insulin secretion, injured pancreatic cells with altered carbohydrate, lipid, and protein metabolism and moreover increased risk of complication of many diseases. According to the report generated by World health organization (WHO), in 2014 approximately 422 million adults were living with diabetes globally, in comparison to 108 million in 1980. The worldwide occurrence of diabetes has almost doubled since 1980, increasing from 4.7 to 8.5% in the adult population. Over the past decade, diabetes incidence has increased faster in low and middle-income countries than in high-income countries[1]. According to the International Diabetes Federation, in 2013 around 50% of all people with diabetes live in just three countries: China consists 98.4 million, India having 65.1 million and the USA 24.4 million.

Therapy of diabetes includes long term use of oral hyperglycemic agents or insulin therapy and lifestyle modifications, dietary control and regular physical exercise[2]. In recent times it has been reported that phytotherapy is considered to be less toxic and with minimal or no side effects in comparison with allopathic medicines[3]. Reports from ethnobotany suggested that about 800 medicinal plants possess an anti-diabetic potential and bioactive compounds[4]. Various botanical medicinal plants have been found significantly managing diabetes and among them, *Leucas aspera* is a significant one.

In our earlier investigations evidenced that the biological approach of synthesis of Iron oxide nanoparticles using *Leucas aspera* leaf extract was an eco-friendly and cost-effective alternative to conventional chemical and physical methods and would be suitable for developing large scale production[5]. In further, to use this comfortable, economic and greener method for development and application of iron oxide nanoparticles in environmental and biomedical requirements; in this present *in-vivo* study, we evaluated the pharmacological studies, enzymatic and non-enzymatic tests and assessed anti-diabetic potential of *Leucas aspera* leaf extract in streptozotocin-induced diabetic Wistar albino rats to prove the effectiveness.

MATERIALS AND METHODS

***In-vitro* studies:**

An eco-friendly synthesis of iron oxide nanoparticles and characteristics of the obtained Fe_3O_4 nanoparticles were studied in detail in the earlier published methodology[5] for Ultraviolet-visible spectroscopy (UV-Vis), Fourier Transform Infra-Red Spectroscopy (FTIR), Scanning Electron Microscope (SEM), Energy-dispersive X-ray spectroscopy (EDX), X-Ray Diffraction (XRD) and High-Performance Liquid Chromatography (HPLC). The synthesized Iron oxide nanoparticles were also utilized for the antibacterial activity and antioxidant studies[6].

***In-vivo* studies:**

Animals

Wistar albino rats of either sex weighing between 150-200 gm were used for this study. The animals were obtained from animal house, Nandha College of Pharmacy, Erode. On arrival, the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2°C and relative humidity of 30 – 70 %. A 12:12 light: day cycle was followed. All animals were allowed to free access to water and fed with standard commercial pelleted rat chaw (M/s. Hindustan Lever Ltd, Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethics Committee (688/PO/Re/S/02/CPCSEA) and were in accordance with the Institutional ethical guidelines.

Experimental Induction of Diabetes in Rats

Diabetes was induced experimentally in 12 hours fasted rats by a single intraperitoneal injection of Streptozotocin (50mg/kg) dissolved in 0.1M of citrate buffer (pH 4.5), followed by intraperitoneal administration of Streptozotocin (120 mg/kg) after 15 minutes. Since STZ is capable of inducing fatal hypoglycemia due to the sudden marked release of insulin from the pancreas, the rats that had been administered STZ were provided after 6 hours with a 10% glucose solution orally for 24 hours continuously so as to prevent hypoglycemia. After 72 hours, rats with a blood glucose concentration above 200 mg/dl were considered to be diabetic and were used for further diabetic studies.

Experimental Design

After the successful induction of experimental diabetes, the rats were divided into five groups of each five rats. Group I (Control 0.1% CMC): Normal rats received 0.1 % Carboxy Methyl Cellulose Solution (1mg/kg) as through oral route. Group II (Diabetic Control): Rats with STZ - Streptozotocin-induced diabetic that were left untreated. Group III (Reference Control): Rats with STZ - Streptozotocin-induced diabetic rat treated with Glibenclamide (5mg/kg). Group IV (Compound C 200): Rats with STZ - Streptozotocin-induced diabetic rat treated with test Compound 200mg/kg of *Leucas aspera* leaf extracted in hot water. Group V: (Compound C 400): Rats with STZ - Streptozotocin-induced diabetic rat treated with test Compound 400mg/kg of *Leucas aspera* leaf extracted in hot water. All the test compounds and the reference drug Glibenclamide administered once daily for 14 days through an oral route using gastric gavage tubes.

Blood was withdrawn from the tail vein on 0, 4th, 7th and 14th day of drug administration and glucose levels are measured using a glucometer. At the end of the experimental period, the rats were fasted overnight, anesthetized with Pentobarbitone sodium (60mg/kg, i.p) anesthesia and the blood was collected by a retro-orbital puncture in non-heparinized tubes. To obtain serum, blood samples were placed at room temperature for 30 minutes and centrifuged at 3000 X *g* for 10 minutes and the supernatant was taken for the determination of Lipid profiles, Liver function (AST, ALT, ALP) and Kidney function test.

Preparation of Homogenate

The animals were sacrificed with excess pentobarbitone sodium and the liver was removed from the animal and kept in the 0.2M, pH 6.6, phosphate buffer. The liver was crushed and the obtained homogenate was subjected to centrifugation process and centrifuged at 3000 rpm for 10 min. The obtained supernatant was used for enzymatic and non-enzymatic tests. Determination of non-enzymatic antioxidant estimation of reduced glutathione (GSH) was measured by the method[7] described by Beutler *et al*., 1986.

Determination of enzymatic anti-oxidants tests were studied as per the standard protocols such as Catalase (CAT)[8], Superoxide dismutase (SOD)[9], Glutathione reductase (GSSH)[10], Peroxides (Px) activity[11], Glutathione peroxidase (GPx) activity was measured by the method described by Paglia and Valentine[12]. Estimation of lipid hydroperoxides[13] and estimation of malondialdehyde (MDA) analysis for lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) and hydroperoxides (HP) were measured by the method of Nieshus and Samuelsson[13].

Estimation of Serum Creatinine[14] was studied and absorbance was read at 520 nm and concentration of serum creatinine in mg/dl was calculated. Similarly, Protein content of the tissue homogenate was assayed by the method of Lowry[15] and absorbance was measured at 660 nm. Protein content was expressed as µg/mg of protein. Estimation of Blood Urea[16] test was studied up to 14 days and the absorbance was read at 540 nm and concentration of urea in mg/dl was calculated.

Determination of serum Lipid Profile

Serum lipid profiling was studied as per the standard protocols, the serum sample were subjected to lipid analysis, such as Total cholesterol (TC), Triglycerides (TG), high-density lipoprotein, cholesterol (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein cholesterol (VLDL).

Statistical Analysis

Results were represented as mean ± SEM. The data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's't' test using GraphPad version 3. P values < 0.05 were considered as significant.

RESULTS AND DISCUSSION

Recent developments in nanotechnology have a led to the growth of novel nanomaterial, which ultimately increases possible health and environmental hazards. Attention in developing environmentally benign procedures for the synthesis of nanoparticles has been improved. The purpose is to minimize the negative impacts of synthetic procedures, their accompanying chemicals, and derivative compounds. The exploitation of different biomaterials for the synthesis of nanoparticles is considered a valuable approach in green nanotechnology[6].

For evidence, Iron oxide nanoparticles using leaf extract of *Leucas aspera* was investigated in detail in the earlier *in-vitro* study. The characteristics of the obtained Fe_3O_4 nanoparticles were studied using UV-Visible spectrophotometer, FTIR, SEM with EDX, XRD, and HPLC.

The synthesized Iron oxide nanoparticles were successfully exploited for the antibacterial activity and antioxidant studies[5]. To prove the efficacy of the anti-diabetic potential of *Leucas aspera* leaf extract in *the in-vivo* model, we evaluated the anti-diabetic potential of *Leucas aspera* leaf extract in Streptozotocin-induced diabetic Wistar albino rats to attest the potency and effectiveness.

The effect of the test compound from leaf extracts of *Leucas aspera* was studied for its hypoglycemic effect against Streptozotocin-induced diabetic rats and the results are tabulated in Table 1. The induction of diabetes in rats was confirmed by the elevation of blood sugar levels after the administration of STZ more than 200 mg/dl. Streptozotocin-induced diabetic rat treated with Glibenclamide (5mg/kg) was used as reference control significantly reduced the blood sugar levels from the 4th day onwards and on the 14th day, the blood sugar was found to be 106.60± 8.43 mg/dl. The 200 mg of hot water extract of *L. aspera* leaf moderately decreased the blood sugar level 117.66±4.49 from 4th day onwards and the effect was maintained until the end of the treatment against the reference control (106.60± 8.43 mg/dl). The 400 mg/kg of hot water extract of *L. aspera* leaf showed (98.35±7.73) good hypoglycemic activity against STZ induced diabetes in rats.

Table 1. Effect of test compounds on Blood Sugar Levels of STZ induced Diabetic in Rats

Drug Treatment	Mean Blood Sugar Level (mg/dl)					
	Before STZ	After STZ	0 Day	4th Day	7th Day	14th Day
Control 0.1% CMC	103.66± 6.65	100.30± 7.76	104.54± 7.33	98.71± 6.88	97.54± 6.92	103.83± 8.72
Diabetic Control	99.53± 5.31	223.59± 9.35	221.62± 12.60	229.34± 9.52	220.51± 5.83	227.58± 8.34
Reference Control	103.16± 7.41	217.32± 8.71	210.44± 11.96	182.05± 8.62**	127.28± 8.50***	106.60± 8.43***
Compound C 200	91.50± 6.30	219.00± 9.22	209.30± 4.62	196.58± 4.70	164.20± 5.07**	117.66± 4.49***
Compound C 400	115.71± 5.90	216.37± 7.40	211.00± 6.35	163.22± 5.44**	137.61± 9.90***	98.35± 7.73***

Values are in mean ± SEM (n=5), *P<0.05, **P<0.01, ***P<0.001 Vs Diabetic Control

The effect of leaf extracts of *Leucas aspera* on serum sample were studied to lipid analysis and biochemical parameters like Total cholesterol (TC), Triglycerides (TG), High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL) and Very Low-Density Lipoprotein (VLDL) were analyzed and the results are summarized and biochemical parameters analysis is tabulated in Table 2.

The animals treated with STZ enhanced the total cholesterol compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the total cholesterol (163.24± 8.04) enhanced by STZ (124.40± 10.63). The low dose (200 mg/kg) of hot water leaf extract of *L. aspera* showed less significant (P<0.05) decrease in cholesterol by 141.65± 7.05 and the high dose (400 mg/kg) of hot water leaf extract of *L. aspera* showed more significant (P<0.01) decrease in cholesterol by 131.24± 6.82 as compared to diabetic control (163.24± 8.04).

The animals treated with STZ enhanced the triglycerides compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the triglycerides (121.75± 7.62) enhanced by STZ (69.27±5.88). The low dose (200 mg/kg) of hot waterleaf extract of *L. aspera* showed less significant (P<0.01) decrease in triglycerides by 84.65±4.52 and the high dose (400 mg/kg) of hot water leaf *L. aspera* leaf extracts showed more significant (P<0.01) decrease in triglycerides by 77.74±6.30 as compared to diabetic control (121.75± 7.62).

The animals treated with STZ enhanced HDL Cholesterol compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the HDL Cholesterol (21.54± 1.86) enhanced by STZ (38.65±2.54). The low dose (200 mg/kg) of hot waterleaf extract of *L. aspera* showed less significant (P<0.05) decrease in HDL Cholesterol by 25.53±1.44 and the high dose (400 mg/kg) of hot water leaf *L. aspera* leaf extracts showed more significant (P<0.001) decrease in HDL Cholesterol by 32.80±1.90 as compared to diabetic control (21.54± 1.86).

The animals treated with STZ enhanced LDL Cholesterol compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the LDL Cholesterol (79.33± 6.24) enhanced by STZ (46.30±2.52). The low dose (200 mg/kg) of hot waterleaf extract of *L. aspera* showed less significant (P<0.05) decrease in LDL Cholesterol by 65.63±3.42 and the high dose (400 mg/kg) of hot water leaf *L. aspera* leaf extracts showed more significant (P<0.01) decrease in LDL Cholesterol by 55.43±2.11 as compared to diabetic control (79.33± 6.24).

The animals treated with STZ enhanced VLDL Cholesterol compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the VLDL Cholesterol (34.40± 2.76) enhanced by STZ (18.39±1.12). The low dose (200 mg/kg) of hot waterleaf extract of *L. aspera* showed less significant (P<0.05) decrease in VLDL Cholesterol by 29.37±2.03 and the high dose (400 mg/kg) of hot water leaf *L. aspera* leaf extracts showed more significant (P<0.001) decrease in VLDL Cholesterol by 23.22±1.09 as compared to diabetic control (34.40± 2.76).

Table 2. Effect of test compounds on Lipid Levels of STZ induced Diabetic in rats

Drug Treatment	Lipid Profiles (mg/dl)				
	Total Cholesterol	Triglyceride	HDL - Cholesterol	LDL - Cholesterol	VLDL Cholesterol
Control 0.1% CMC	117.63± 8.52	65.21± 3.64	40.27± 2.95	41.34± 2.55	16.97± 1.08
Diabetic Control	163.24± 8.04	121.75± 7.62	21.54± 1.86	79.33± 6.24	34.40± 2.76
Reference Control	124.40± 10.63**	69.27± 5.88***	38.65± 2.54***	46.30± 2.52***	18.39± 1.12***
Compound C 200	141.65± 7.05*	84.65± 4.52**	25.53± 1.44*	65.63± 3.42*	29.37± 2.03*
Compound C 400	131.24± 6.82**	77.74± 6.30**	32.80± 1.90***	55.43± 2.11**	23.22± 1.09***

Values are in mean ± SEM (n=6), *P<0.05, **P<0.01, ***P<0.001 Vs Diabetic Control

The effects of leaf extracts of *L. aspera* leaves on serum blood urea nitrogen (BUN) and creatinine in STZ induced diabetes in rats and the results were tabulated in table 3. The Blood Urea Nitrogen and serum creatinine was increased in the STZ induced diabetes animals as compared to normal control animals. Hot water extract of *L. aspera* leaf at a dose of 200 mg/kg showed less significant (P<0.001) decrease in blood urea nitrogen by 22.54± 1.65 and at 400 mg/kg dose of hot water extract of *L. aspera* leaf more significant (P<0.001) decrease in blood urea nitrogen was observed as 20.58 ± 1.44 as compared to diabetic control (39.63 ± 2.63). Similarly, hot water extract of *L. aspera* leaf at a dose of 200 mg/kg showed less significant (P<0.001) decrease in creatinine by 0.77± 0.02 and at 400 mg/kg dose of hot water extract of *L. aspera* leaf more significant (P<0.001) decrease in creatinine was observed as 0.69 ± 0.03 as compared to diabetic control (1.84 ± 0.01).

Table 3. Effect of test compounds on Kidney functions of STZ induced Diabetic in rats

Drug Treatment	Kidney Function Test	
	BUN (mg/dl)	Creatinine (mg/dl)
Control 0.1% CMC	17.35±0.96	0.48±0.02
Diabetic Control	39.63±2.63	1.84±0.01
Reference Control	19.66±1.04***	0.63±0.03***
Compound C 200	22.54±1.65***	0.77±0.02***
Compound C 400	20.58±1.44***	0.69±0.03***

Values are in mean ± SEM (n=6), *P<0.05, **P<0.01, ***P<0.001 Vs Diabetic Control

The effects of leaf extracts of *L. aspera* leaves on MDA and LH in STZ induced diabetes in rats and the results were tabulated in table 4. The MDA and LH were increased in the STZ induced diabetes animals as compared to normal control animals. Hot water extract of *L. aspera* leaf at a dose of 200 mg/kg showed a decrease in MDA by 1.28± 0.03 and at 400 mg/kg dose of hot water extract of *L. aspera* leaf more significant (P<0.05) decrease in MDA was observed as 1.02 ± 0.04 as compared to diabetic control (1.32 ± 0.02). Similarly, hot water extract of *L. aspera* leaf at a dose of 200 mg/kg showed less significant (P<0.01) decrease in LH by 1.01± 0.04 and at 400 mg/kg dose of hot water extract of *L. aspera* leaf more significant (P<0.05) decrease in LH was observed as 0.94 ± 0.05 as compared to diabetic control (1.08 ± 0.02).

Table 4. Effect of test compounds on liver MDA and LH in control and experimental animals

Drug Treatment	MDA (nmoles/min/mg protein)	LH (nmoles/min/mg protein)
Control 0.1% CMC	0.78±0.03	0.64±0.04
Diabetic Control	1.32±0.02[a]	1.08±0.02[a]
Reference Control	0.99±0.01[b]	0.87±0.01[b]
Compound C 200	1.28±0.03	1.01±0.04[b]
Compound C 400	1.02±0.04[c]	0.94±0.05[c]

Values are mean ± SEM; n=6 in each group; [a]P <0.01 when compared to normal control; [b]P<0.01, [c]P<0.05, when compared to diabetic control (one-way ANOVA followed by Dunnett's 't' test).

The effects of leaf extracts of *L. aspera* leaves on liver enzymatic antioxidants in STZ induced diabetes in rats and the results were tabulated in table 5. The Catalase (CAT), Superoxide dismutase (SOD), Glutathione reductase (GSSH), Peroxides (Px) activity, Glutathione peroxidase (GPx) activity results were observed that effect was increased in the STZ induced diabetes animals as compared to normal control animals. Similarly, the effects of leaf extracts of *L. aspera* leaves on liver non-enzymatic antioxidants in STZ induced diabetes in rats and the results were tabulated in table 6. The reduced glutathione (GSH), Vitamin C and Vitamin E results were observed that effect was also increased in the STZ induced diabetes animals as compared to normal control animals.

Table 5. Effect of test compounds on liver enzymatic antioxidants in control and experimental animals

Drug Treatment	CAT (µmoles/min/mg protein)	SOD (nmoles/min/mg protein)	GSSH (nmoles/min/mg protein)	Px (nmoles/mg protein)	GPx (nmoles/mg protein)
Control 0.1% CMC	42.1±1.8	5.1±0.06	34.8±1.2	7.6±0.02	10.2±0.03
Diabetic Control	30.6±1.4[a]	3.6±0.04[a]	21.9±0.8[a]	6.2±0.03[a]	7.8±0.02[a]
Reference Control	41.3±2.1[b]	4.8±0.05[b]	32.6±0.9[b]	7.3±0.02[b]	9.7±0.06[b]
Compound C 200	36.3±0.9[b]	3.6±0.04	25.6±0.8[b]	6.5±0.03[c]	8.3±0.05[b]
Compound C 400	39.6±1.2[b]	3.9±0.07[c]	28.1±0.9[b]	6.8±0.04[b]	8.9±0.02[b]

Values are mean ± SEM; n=6 in each group; [a]P <0.01 when compared to normal control; [b]P<0.01, [c]P<0.05, when compared to diabetic control (one-way ANOVA followed by Dunnett's 't' test).

Table 6. Effect of test compounds on liver non-enzymatic antioxidants in control and experimental animals

Drug Treatment	GSH (nmoles/min/mg protein)	Vitamin C µgm/mg protein	Vitamin E µgm/mg protein
Control 0.1% CMC	12.4±0.9	4.8±0.03	7.1±0.02
Diabetic Control	9.6±0.8[a]	3.4±0.02[a]	5.8±0.03[a]
Reference Control	11.8±0.7[b]	4.5±0.04[b]	6.9±0.02[b]
Compound C 200	10.2±0.5[b]	3.3±0.03	5.9±0.03
Compound C 400	10.8±0.6[b]	3.9±0.02[b]	6.5±0.04[b]

Values are mean ± SEM; n=6 in each group; [a]P <0.01 when compared to normal control; [b]P<0.01, [c]P<0.05, when compared to diabetic control (one-way ANOVA followed by Dunnett's 't' test).

Thus collectively, the current study examined the pattern of diabetes induced by chemical agent Streptozotocin and oral glucose loading in experimental rats, and their responses to anti-diabetic agents in order to deduce the suitability or otherwise of chemical induction as a model for studying diabetes mellitus in experimental animals was investigated positively.

CONCLUSION:

Though different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, there is a growing interest in herbal remedies, due to the side effects associated with these therapeutic agents. A need is felt to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to the public. This can be achieved by screening newer molecules or plant products, which may be effective at non-toxic dose levels. In the Siddha and Ayurveda system of treatment, dry powder or crude extracts of plants are used to treat various disorders including cancer. The observed effect is attributed to may be a single compound or combination of compound present in the crude extracts. Because of perceived effectiveness, minimal side effects in clinical experience and relatively low cost, herbal drugs are widely prescribed even when their biologically active compounds are unknown. Our study proved in such a way by *in-vitro* and *in-vivo* to explore subsequent free radical scavenging and anti-diabetic effect of the traditionally used plant, *Leucas aspera*. This present study thus will become a milestone in developing a new drug from unexplored plant leaves, *Leucas aspera* for utilization of the human population in an effective manner for the control of the diabetes mellitus.

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